Effect of Endothelial Differentiated Adipose-Derived Stem Cells on Vascularity and Osteogenesis in Poly(D,L-Lactide) Scaffolds In Vivo

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Abstract: Prevascularization of engineered bony constructs can po tentially improve in vivo viability. However, the effect of endothelial cells on osteogenesis is unknown when placed in poly(D,L lactide) (PLA) scaffolds alone. Adipose derived stem cells (ASCs) have the ability to differentiate into both osteoblasts and endothelial cells by culture in specific media. We hypothesized that ASC derived endo thelial cells would improve vascularity with minimal contribution to bone formation when placed in scaffold alone. ASCs were suc cessfully differentiated into endothelial cells (ASC Endo) and os teoblasts (ASC Osteo) using media supplemented with vascular endothelial growth factor and bone morphogenic protein 2, respec tively. Tissue engineered constructs were created with PLA ma trices containing no cells (control), undifferentiated ASCs (ASCs), osteogenic differentiated ASCs (ASC Osteo), or endothelial differ entiated ASCs (ASC Endo), and these constructs were evaluated in critical size Lewis rat calvarial defect model (n 34). Eight weeks after implantation, the bone volume and microvessel population of bony constructs were evaluated by micro computed tomography analysis and histologic staining. Bone volumes for ASCs and ASC Osteo constructs, 0.7 and 0.91 mm^3, respectively, were statistically greater than that for ASC Endo, 0.28 mm^3 ($P < 0.05$). There was no statistical difference between the PLA control (0.5 mm^3) and ASC Endo (0.28 mm^3) constructs in bone formation. The percent area of microvessels within constructs was highest in the ASC Endo group, although it did not reach statistical significance (0.065). Prevascu larization of PLA scaffold with ASC Endo cells will not increase bone formation by itself but may be used as a cell source for improving vascularization and potentially improving existing osteoblast function.

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Reconstruction of large bony defects due to trauma, neoplastic resection, and pediatric craniofacial deformity is a challenging problem in reconstructive surgery. Autologous free tissue transfer remains the current criterion standard for treating these large de fects. However, donor site morbidity and limited tissue supplies are major limitations of such procedures. An increasingly attrac tive solution is the use of stromal stem cells for engineering bony constructs, which overcomes current donor site challenges while providing an autologous alternative. Interestingly, fat contains an abundant supply of stromal stem cells and is a tissue most patients already seek to have electively removed. Studies have already shown that use of adipose derived stem cells (ASCs) cultured in osteogenic media can improve the healing of critical size calvarial defects in several species.[1–3] Success, however, is met with new challenges. Tissue engineered bony constructs currently lack an intrinsic blood supply. Once placed in vivo, these engineered tissues rely on dif fusion for nutrients, oxygen, and removal of waste until a capillary network is established. Although diffusion is sufficient to main tain cellular viability in small implants, larger constructs require a mechanism beyond diffusion alone, optimally a functional vascular network, to prevent central necrosis of the bony construct. Pre vascularization of engineered tissue appears to be a promising method for increasing the size of engineered tissue constructs, while keeping cells adequately replenished with nutrients and removing cellular waste.

The stromal vascular fraction (SVF) is the cellular compo nent derived from collagenase digestion of adipose tissue from which ASCs are derived. It contains cells capable of differentiating into multiple phenotypes including adipose, cartilage, osteogenic, smooth muscle, and neuronal lineages.[4–8] Recently, ASCs have been successfully and reliably differentiated into an endothelial cell lineage.[9–11] Stromal vascular fraction contains a subpopulation of $CD31^+$ cells consistent with an endothelial phenotype. This sub population also possesses the capacity to differentiate into an adi pocyte lineage when placed in adipogenic media.[10] Given the relative plasticity and heterogeneity of the SVF, endothelial differ entiated ASCs may have multiple mechanisms to improve bone formation. This cell population may transdifferentiate,[10] improve osteoblast activity via cellular interactions,[12,13] or cause migration of host osteoblasts into the scaffold to increase osteogenesis. Al ternatively, differentiation of ASCs into endothelial cells may be terminal, and its potential role in tissue engineering may be strictly to improve the function of existing osteoblasts in scaffolds.

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In vivo studies have shown mesenchymal stem cells differentiate into endothelial cells, improve osteogenesis, increase vascularization, and minimize central necrosis in osteoblast seeded scaffolds.[14] Although improved vascularization of seeded osteoblasts within the engineered tissue is thought to be the driving force behind improved osteogenesis and decreased central necrosis, to what extent neovascularization alone is responsible for increased osteogenesis is not clear. The mechanism may potentially involve either interplay between endothelium and osteoblasts, infiltration of host stem cells/osteoblasts into the scaffold, improved nutrient supply, or a combination of mechanisms. The increased vascularization of a scaffold in itself may potentially increase ossification within the engineered scaffold without the addition of osteoblasts.

A successful scaffold for bone regeneration promotes vascularization while allowing for cell attachment, infiltration, and migration, to form functional bone. Poly(D,L lactide) (PLA) is a biocompatible and biodegradable polymer that has been used in a multitude of implantable biomedical devices, including tissue engineering scaffolds.[15] Its limitation as an implant biomaterial mostly stems from its hydrophobic nature. It has been shown that cell adhesion and proliferation on polymeric scaffolds are affected by surface characteristics such as wettability, surface chemistry, and roughness. Glow discharge gas plasma (GP) treatment is a surface treatment that introduces modifications of polymers to enhance surface cell adhesion and growth. It has been shown that GP treated PLA has the potential to serve as angiogenic scaffolds.[16] Because previous work with endothelial cells on GP treated scaffolds show increased cell proliferation at 4 to 6 days and increased vascularization of the PLA scaffold in vivo at 12 days compared with non treated PLA scaffolds, the use of GP treated PLA scaffold has the potential to serve as an excellent angiogenic composite.[16]

The goals of this study were to demonstrate differentiation of Lewis rat ASCs into an endothelial lineage and to compare in vivo bone and vessel formation between ASCs, ASCs differentiated into osteoblastic lineage, and ASCs differentiated into endothelial lineage when seeded on PLA scaffolds. We hypothesized that ASCs derived endothelial cells would improve vascularity with minimal contribution to bone formation when placed alone in vivo in scaffold.

MATERIALS AND METHODS

ASC Isolation and Culture

Following an Institutional Animal Care and Use Committee approved protocol, 2 month old Lewis rats were anesthetized using isoflurane. The abdominal area was then shaved and prepared with Betadine. Using sterile technique, a 3 to 4 cm midline abdominal incision was introduced for access. After identification of the kidneys and testes, the perinephric and peritesticular adipose tissue was removed with sharp dissection. Following removal, the adipose tissue was placed in sterile pH balanced Hanks balanced salt solution containing 1% bovine serum albumin for further processing. The animals were immediately killed via intracardiac injection of 0.5 mL Fatal Plus (Voretch Pharmaceuticals, Dearborn, MI) and disposed of following vivarium regulations.

The adipose tissue was finely minced using sharp dissection and centrifuged for 5 minutes at 500*g*. The free floating fat was removed, and remaining tissue/cell suspension was placed into 25 mL of 1% bovine serum albumin Hanks balanced salt solution containing collagenase (200 U/mL) and allowed to agitate at 37°C for 1 hour. The digest was filtered through 100 μm nylon mesh twice and then centrifuged for 5 minutes at 500*g*. The supernatant was removed, and the pellet resuspended in MesenPro media (Invitrogen, Carlsbad, CA) to a concentration of 4×10^4 cells/mL, plated overnight, and incubated at 37°C with 5% CO_2. Cells were used for differentiation after the third passage.

PLA Scaffolds

Poly(D,L lactide) scaffolds were fabricated using a vibrating particle salt leaching method, which yields an open cell construct with interconnected pores, porosity of 90%, and pores 250 to 600 μm in diameter.[17] Poly(D,L lactic) polymer (DURECT Corp, Birmingham, AL) was dissolved in acetone, and the polymer solution was added to sodium chloride and vibrated under continuous flow conditions. Eight millimeter diameter disks of 1.2 mm thickness were punched out of the polymer mixture after drying, and the sodium chloride was leached out in sterile deionized water. After 2 days in water, scaffolds were lyophilized. Before seeding, GP treatment was performed in a pure oxygen environment in a glow discharge system (PDC 32G, Plasma Cleaner/Sterilizer; Harrick Scientific Inc, Pleasantville, NY) for 3 minutes at 100 W as a surface treatment and for sterilization.

Seeding Scaffolds

Before seeding, the scaffolds were degassed in culture media under vacuum and placed in a 24 well ultralow attachment tissue culture plate (Corning Life Sciences, Lowell, MA). Cells were resuspended to a concentration of 100,000 cells per 20 μL before being added dropwise to the scaffold surface. The cells were allowed to incubate for 30 minutes at 37°C 5% CO_2 before adding 500 μL of the culture media to each well.

Endothelial Differentiation of ASCs

Differentiation

After the third passage, cells were seeded on the PLA scaffolds with Dulbecco modified Eagle medium, 2% fetal calf serum (FCS), and 50 ng/mL recombinant rat vascular endothelial growth factor C (VEGF C; Promocell; Heidelberg, Germany). The media was changed 3 times weekly for 8 days before implantation in polymer scaffolds. Vascular endothelial growth factor C was added fresh to stock media before each media change. Differentiation was confirmed by immunocytochemistry and angiogenesis assay.

Immunocytochemistry

Endothelial differentiated ASCs (ASC Endo) were plated on chamber slides (Nunc Lab Tek, Rochester, NY) at 20,000 cells per chamber for 24 hours in endothelial differentiation media. Cells were fixed in 100% methanol for 5 minutes at −20°C and allowed to air dry, followed by 3 brief washes in phosphate buffered saline (PBS) with pH of 7.2. Cells were then permeabilized with 0.5% Triton C 100 for 10 minutes, followed by 4 washes with PBS. Cells were blocked with goat serum (Vector Laboratories, Burlingame, CA) diluted in 10 mL of PBS for 20 minutes. Cells were incubated with primary antibodies against PECAM 1 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight. After rinsing with PBS for 5 minutes, Alexa Fluor 488 (Life Technologies, Grand Island, NY) secondary antibodies were added for 1 hour. Chamber slides were then disassembled, and 1 drop per chamber of VectaShield containing 200 ng/mL of DAPI was used to mount cover slips, and the slides were stored in a dark room overnight. Cells were visualized under fluorescence microscopy at 20× and 40×.

Western Blotting Analysis for von Willebrand Factor Expression in ASCs

Adipose derived stem cells were grown on 100 mm dishes at 70% confluency per dish and were treated with 50 ng VEGF C for 7 days. Whole cell extracts were prepared by lysing cells in 500 μL RIPA lysis buffer/dish. Proteins were quantitated using a BCA assay

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(Pierce, Rockford, IL) and fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to poly vinylidene fluoride membrane at 4°C for 2 hours. The blots were probed with anti von Willebrand factor (anti vWF) (Santa Cruz) at 1:500 dilutions overnight at 4°C. The proteins were detected by chemiluminescence according to manufacturer's recommendations (ECL; Amersham, Arlington Heights, IL). Actin was used as an internal control. Each experiment was repeated 3 times.

Angiogenesis Assay

After ASCs were differentiated with endothelial differen tiation media, 5×10^3 cells in 50 μL were plated on a gel matrix (Chemicon; Temecula, CA) in a 96 well plate. After 1 hour of in cubation at 37°C, cells were examined via microscope for capillary formation. Undifferentiated ASCs were also plated on the gel matrix as a control.

Osteogenic Differentiation of ASCs

Differentiation

After the third passage, cells were seeded on the PLA scaf folds and incubated with osteogenic media consisting of α MEM, 10% fetal bovine serum, dexamethasone (0.1 μM), ascorbate 2 phosphate (0.05 M), β glycero 2 phosphate (1000 μM), and BMP 2 (10 ng/mL). The medium was changed 3 times weekly for a total of 21 days. Differentiation was confirmed by immunocytochemistry.

Immunocytochemistry

Osteogenic differentiated ASCs (ASC Osteo) were trypsinized from the PLA scaffolds and plated on chamber slides for 24 hours in osteogenic media at 20,000 cells per chamber. Cells were fixed in 100% methanol for 5 minutes at −20°C and allowed to air dry, fol lowed by 3 brief washes in PBS with pH 7.2. Cells were then per meabilized with 0.5% Triton X 100 for 10 minutes, followed by 4 washes with PBS. For osteopontin (OPN), cells were blocked with goat serum (Vector Laboratories) diluted in 10 mL of PBS for 20 minutes. For osteocalcin (OCN), donkey serum was used. After 1 rinse, cells were incubated with primary antibody, OPN (73631; Santa Cruz) or OCN (30044; Santa Cruz) at 1:50 dilution overnight. After a 5 minute rinse with PBS, secondary antibodies, Alexa Fluor for OPN and Texas red for OCN, were added for 1 hour. After a brief rinse, chamber slides were disassembled, and 1 drop per chamber of VectaShield containing 200 ng/mL of DAPI was used to mount cover slips, and the slides were stored in a dark room overnight. Cells were visualized under fluorescence at 20× and 40×.

Viability Assay

Cells seeded onto the scaffolds were visualized using the Live/Dead assay (Invitrogen) 24 hours after seeding. The scaffolds were gently washed with PBS to remove nonadherent cells, then incubated with Live/Dead assay (Invitrogen) at a concentration 2 mM calcein AM and 4 mM ethidium homodimer in PBS for 30 minutes, which labeled live cells green and dead cells red, re spectively. The scaffolds were again gently washed with PBS before imaging using a fluorescence microscope at 10×.

Proliferation Assay

Proliferation of ASCs, ASC Endo, and ASC Osteo in the scaffolds was measured on days 1, 3, 7, 12, 15, and 21. AlamarBlue (Invitrogen) assay was used to monitor proliferation. At each time point, the scaffolds were incubated for 1.5 hours at 37°C with 5% CO_2 in 500 μL of a 10% AlamarBlue solution in a 24 well plate. After incubation, the scaffolds were removed from the well plates, rinsed with PBS, transferred to a new plate, and placed back in the incubator for further culture. Fluorescence was measured using a fluorescence plate reader at excitation at 540 nm and emission at 590 nm.

Metabolic Activity

Metabolic activity was also measured in all the scaffolds before they were implanted into the animals. The AlamarBlue assay was performed in the same manner as the proliferation assay.

In Vivo Assay

Lewis rats were anesthetized using isoflurane. The scalps were shaved and prepared for surgery. A sagittal incision was made over the midline scalp through the periosteum and reflected. Using a trephine burr, an 8 mm defect was created (n 34) in the calvaria (Fig. 1). The bone flap was removed with extreme care to minimize dural tears. The calvarial defects were randomly filled with 4 dif ferent scaffolds: (1) PLA scaffold alone (control, n 9), (2) PLA scaffold + ASC Endo (Endo, n 8), (3) PLA scaffold + undiffer entiated ASCs (ASC ud, n 8), and (4) PLA scaffold + ASC Osteo (Osteo, n 9). The periosteal flaps were placed over the implants, and the scalp was closed in a single layer of 4 0 Prolene.

Micro-Computed Tomography Imaging

Animals were killed after 8 weeks of implantation, and the calvarium removed and placed in formalin. Micro computed to mography (micro CT) analyses of all samples were performed be fore histology using Skyscan 1076 (Skyscan, Kontich, Belgium) at an 8.77 μm pixel resolution while hydrated in formalin. The micro CT reconstructed axial slices were then evaluated using standard morphometric measures using CTAn software (Skyscan) to deter mine total mineralization (bone) of the scaffold in vivo. New bone formation was standardized between samples by evaluating a three dimensional volume equivalent to the created bony defect (column with diameter of 8.0 mm and height of 1.3 mm) placed at the center of individual sample defects through three dimensional reconstruction and then counting total voxels with attenuation values corresponding to osteoid/bone.

Histology

After micro CT, the entire section of calvaria was fixed in 10% buffered formalin, decalcified for 25 to 30 minutes in rapid decalcifying solution (TBD 1, Thermo Scientific, Waltham, MA), processed routinely and embedded in paraffin, sectioned in 5 μm slices, and stained with hematoxylin eosin (H&E) and Masson tri chrome stains. Microvessel density was evaluated using ImageJ (National Institutes of Health, Bethesda, MD). Because paraffin embedding process dissolves remaining PLA scaffold, frozen sec tions were compared with paraffin embedded sections to ensure that the voids seen on the paraffin section represented the scaffold.

Statistical Analysis

Continuously distributed outcomes were summarized with the mean ± 1 SD in the text and tables and with the mean ± 1 SEM in plots. Treatment groups were contrasted on the outcome mean using analysis of variance with multiple comparisons corrected for

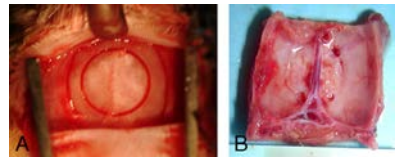

FIGURE 1. In vivo assay and tissue harvest. After anesthesia, an 8-mm defect was created with trephine (A) through a midline scalp incision. Eight weeks later, the calvarium is harvested for analysis (B). Note the disk shape outline of PLA scaffold and a visible intact sagittal sinus.

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using a Tukey test. The significance of changes in the mean from day 1 to day 21 was assessed with a paired *t* test. Data were trans formed appropriately before analysis to approximate normality. Statistical analysis was performed using SAS version 9.2 (SAS In stitute, Cary, NC). Testing was 2 sided with a nominal experiment wise significance level of 5%.

RESULTS

Cell Characterization

Endothelial Differentiation

Characterization of differentiated cells was performed using immunocytochemistry and Western blot analysis. After 8 days of differentiation in endothelial differentiation media, ASCs exhibited an increase in CD31 expression (Fig. 2A). Expression of vWF using immunocytochemistry was not achieved. However, Western blot of vWF revealed an increased expression with 1 week of VEGF stimulation normalized to actin expression (Fig. 2B). Finally, the functional characteristics of endothelial differentiated ASCs were established by capillary like networks forming on Matrigel after 8 days of differentiation (Fig. 3). Nondifferentiated ASCs did not exhibit any tubule formation.

Osteogenic Differentiation

After 3 weeks of differentiation in osteogenic media, ASCs were evaluated for expression of osteoblast surface markers. Both OCN and OPN were shown to be prominently expressed in these differentiated cells using immunofluorescence (Fig. 4).

Viability Assay

The Live/Dead assay showed that ASCs, ASC Endo, and ASC Osteo were able to attach to the scaffold and maintain viabil ity (Fig. 5A).

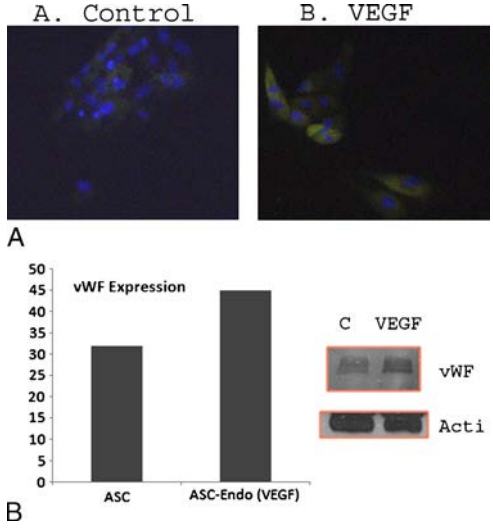

FIGURE 2. A and B, Endothelial differentiation markers. Adipose-derived stem cells from Lewis rats were incubated either in DMEM with 2% FCS or DMEM with 2% FCS plus 50 ng/μL of recombinant VEGF for 1 week in chambered slides. CD31 expression (A, B). Cells were fixed with PFA, incubated with PECAM-1 primary antibodies and Alexa 488 goat anti mouse antibodies to detect CD31 expression using immunofluorescent analysis. An increase in CD31 expression is seen in VEGF group (B), compared with control (A). vWF expression (C). Western blotting analysis using anti-vWF at 1:500 dilutions reveals up-regulation of vWF in ADSC-Endo group.

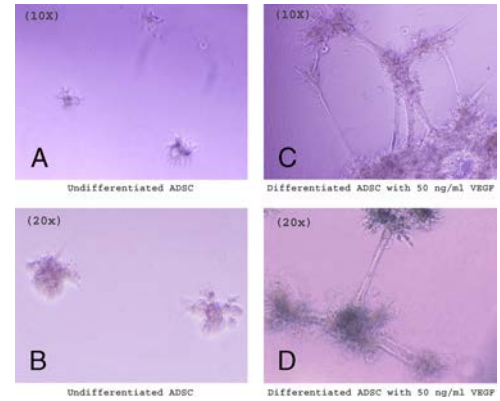

FIGURE 3. Endothelial differentiation and angiogenesis assay. After differentiation of ASCs with VEGF, control and differentiated cells were placed in gel matrix in a 96-well plate for 1 hour at 37°C and examined under microscope in 10× and 20× magnification. Undifferentiated cells (A, B) did not exhibit any tubule formation, whereas VEGF-differentiated cells (C, D) clearly demonstrated capillary-like networks between cells.

Proliferation Assay

At day 1, there were similar numbers of ASCs, ASC Endo, and ASC Osteo. The mean number of ASCs, ASC Endo, and ASC Osteo cells increased from day 1 to day 21, but the increase was significant only for ASC Osteo cells (ASCs [n 3]: day 1: 4115.6 ± 116.0, day 21: 4210.1 ± 159.7 [*P* 0.24]; ASC Endo [n 3]: day 1: 4508.0 ± 109.2, day 21: 5270.6 ± 406.8 [*P* 0.10]; ASC Osteo [n 3]: day 1: 4454.7 ± 405.3, day 21: 5406.4 ± 200.6 [*P* 0.04]).

Metabolic Activity

Metabolic activity of the cells in the scaffold is reflective of the number of viable cells within the scaffold. The mean metabolic activity was significantly increased in ASC ASC Endo and ASC Osteo cells relative to ASCs (ASCs [n 12]: 3182.1 ± 312.2, ASC Endo [n 10]: 4165.6 ± 248.7, ASC Osteo [n 12]: 4399.5 ± 141.4; ASC Endo vs ASCs [$P < 0.001$], ASC Osteo vs ASCs [$P < 0.001$]).

X-ray and Micro-CT Evaluation

Digital radiographs of calvaria extracted after 8 weeks of implantation are presented in Figure 6A. Dispersed areas of bone density are noted in both ASCs and ASC Osteo groups. The PLA alone and ASC Endo group had little, if any, bone spicules visible on digital radiographs. To evaluate quantitative bone formation, micro CT of calvaria was obtained with three dimensional reconstruction (Figs. 6B, C). Consistent with the digital radiographs, ASC and ASC Osteo groups had more bone density compared with that of PLA and ASC Endo group. Using an 8 mm cut, the mean new bone (in mm^3) was significantly increased in ASCs and ASC Osteo cells relative to ASC Endo cells. All other pairwise contrasts were not significant (PLA [n 9]: 0.5 ± 0.43, ASC Endo [n 8]: 0.28 ± 0.21, ASCs [n 8]: 0.7 ± 0.17, ASC Osteo [n 9]: 0.91 ± 0.65; ASC Osteo vs ASC Endo [*P* 0.02], ASC Endo vs ASCs [*P* 0.02]).

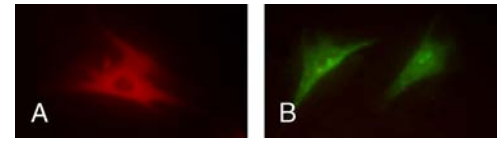

FIGURE 4. Osteogenic differentiation markers. Adipose-derived stem cells were placed in PLA and placed in osteogenic media for 3 weeks. Cells were extracted from PLA and fixed on slide chambers, and immunocytochemistry was performed for OCN (A, Texas red) and OPN (B, Alexa 488).

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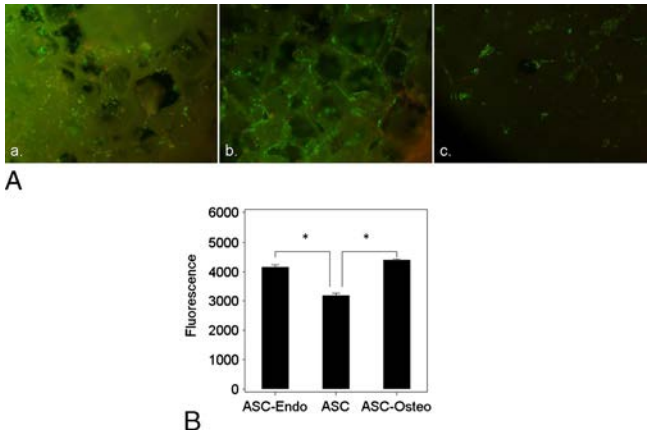

FIGURE 5. Cell viability and metabolic activity assay. Cells seeded onto PLA scaffolds were visualized 24 hours after seeding using Live/Dead assay. Cells from all 3 groups (A) ASCs, (B) ASC-Endo, and (C)ASC-Osteo remained viable as evidenced by green staining. Metabolic activity assay revealed the most activity in ADSC-Osteo, followed by ASC-Endo and lastly by undifferentiated ASCs (D).

Histological Evaluation and Microvessel Density

Hematoxylin eosin histology revealed partial digestion of the PLA scaffold by multinucleated giant cells (Fig. 7A). Microvessels of variable sizes are noted within the infiltrating tissue. Microvessels were counted directly from H&E stained slides under 10× micro scope. The mean percent area (%) with microvessel formation was increased in ASC Endo relative to PLA (Fig. 7B), ASCs, and ASC Osteo; however, the means did not vary significantly with cell type (P 0.16), and all pairwise contrasts were nonsignificant (PLA [n 9]: 0.53 ± 0.23, ASC Endo [n 7]: 1.14 ± 0.52, ASCs [n 8]: 1.03 ± 0.81, ASC Osteo [n 9]: 1.01 ± 0.63; PLA vs ASC Endo [P 0.07]) (Fig. 7B).

DISCUSSION

Lack of a functioning microvascular network is an important hurdle needed to overcome for large engineered tissue to survive in vivo. Currently, the size of engineered tissue is limited to milli meters in order for oxygen and nutrients to adequately reach cells into the most central areas of scaffold via diffusion.[18] Thus, the only viable option readily available for major reconstruction of bony tissues is autologous tissue transfer with a microvascular anastomosis. However, limited donor sites and donor site morbidity make major reconstructions challenging.

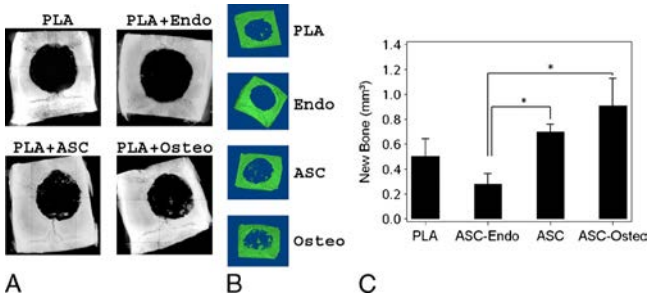

FIGURE 6. A, Bone density, x-ray. Plain x-rays of calvaria extracted immediately after the rats were killed reveal minimal bone formation in PLA and ASC-Endo groups, whereas ASC-ud and ASC-Osteo groups showed significantly increased bone spicules. B and C, Bone density, micro-CT. Analysis of three-dimensional calvarial bone reconstruction (B, C) shows minimal bone formation in PLA and Endo groups, compared with ASC-ud and ASC-Osteo groups.

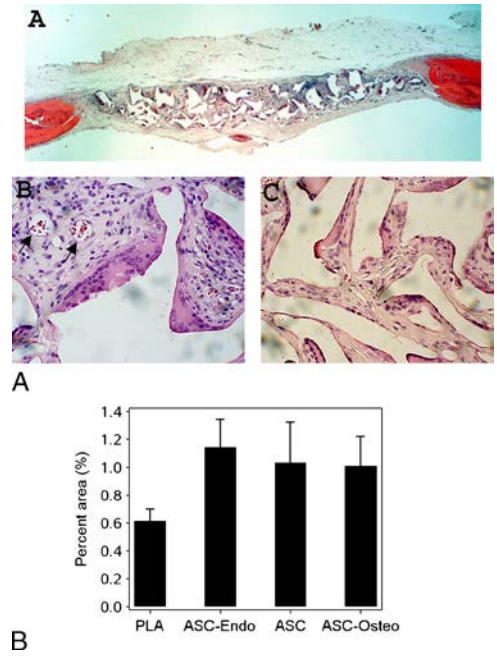

FIGURE 7. A and B, Histological evaluation and microvessel density. Hematoxylin-eosin histology revealed partial digestion of the PLA scaffold by multinucleated giant cells (A). Microvessels of variable sizes are noted within the infiltrating tissue. Microvessels were counted directly from H&E-stained slides under 10× microscope. The mean percent area (%) with microvessel formation was increased in ASC-Endo relative to PLA (B), ASCs, and ASC-Osteo; however, the means did not vary significantly with cell type (P = 0.16), and all pairwise contrasts were nonsignificant (PLA [n = 9]: 0.53 ± 0.23, ASC-Endo [n = 7]: 1.14 ± 0.52, ASCs [n = 8]: 1.03 ± 0.81, ASC-Osteo [n = 9]: 1.01 ± 0.63; PLA vs ASC-Endo [P = 0.07]) (B).

In this study, the presence of ASC Endo in gas plasma treated PLA scaffolds improved in vivo vascularization, especially compared with blank scaffolds, which showed nearly a 2 fold im provement (Fig. 7B). Although this difference approached signifi cance, it did not achieve it (P 0.065). We believe that an increase in the number of animals would resolve this difference. Although not as profound as the ASC Endo treated scaffolds, a similar im provement in vascularization was observed in both the ASC and ASC Osteo treated scaffolds over the blank scaffolds. Acknowl edging the fact that no significant differences were achieved high lights the fact that a large amount of variability was present among the treated scaffolds. Although this is expected among the ASC group, we believe that the large amount of variability in vessel formation among the ASC Osteo group demonstrates to some de gree its retained plasticity. The mechanism by which the vascu larization is potentially improved in ASC and ASC Osteo treated scaffolds is, however, not clear. One plausible mechanism specific to our model may be that the gas plasma treated PLA scaffold, which has previously been shown in vivo to improve vascularization,[16] selects for vessel formation in the presence of less mature cell lines. A pseudoplasticity effect could be, however, explained by the heterogeneity of the SVF from which the ASCs are acquired. This cell population is a mixture of cells with a variety of surface protein expressions with complex potential effects on osteoblasts.[10] Because SVF contain $CD31^+$ cells consistent with endothelial cells as a subgroup of cells (10% from excised and 24% from liposuc tioned adipose tissue),[10] this may partially explain the potential in crease in vascularity in the ASCs and ASC Osteo groups compared with PLA group. A more general explanation may be that the presence of any cells in a scaffold may assist in the formation of a vascular network. Rationally, the initial phase of peripheral to central

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migration of host cells is mitigated, thereby hastening the time to central cells experiencing hypoxic conditioned, if not already being experienced to some degree before implantation. It is well documented that hypoxia induces cells to secrete angiogenic factors that promote angiogenesis.

Interestingly, the increase in vascularization seen in the ASC Endo group did not result in an increase in bone formation. Thus, vascularization of scaffolds in itself is not sufficient to improve osteogenesis, even in smaller scaffolds. Both ASC Osteo and ASC groups had greater than a 2 fold statistically significant increase in bone formation compared with the ASC Endo group. Furthermore, the bone density of the ASC Endo group was almost half of that of the control group, despite being statistically nonsignificant. These results suggest that the ASC Endo may actually lose their plasticity toward an osteogenic lineage. This suggestion assumes that the increased bone formation in the ASC group is due to the plasticity of undifferentiated ASCs favoring an osteogenic lineage in a calvarial defect environment.

The ASC Endo may even have an inhibitory effect on bone formation, given the relative decrease in mineralization over blank scaffolds. This suggestion, however, mandates further study to confirm whether any potential inhibitory effect of ASC Endo on bone formation does indeed exist. Interestingly, our findings parallel those of Seebach et al,[19] who demonstrated comparable results in vivo using a ceramic scaffold seeded with endothelial progenitor cells, a subpopulation found in circulating blood similar to our ASC Endo cells. In either model, a possible source of the bone growth inhibition could be due to contact inhibition from the ASC Endo covering significant surface area of the scaffold and discouraging migration of other cells into the scaffold. There may also be signals from the ASC Endo cells that inhibit inward migration of other cells; however, a specific coculture system with ASC Endo cells and rat primary osteoblasts would have to be investigated to determine if this phenomenon is occurring.

There is coculture evidence against the latter explanation, with reports demonstrating endothelial cells and osteoblasts cultured together in a coculture system improve both in vitro vascularization[20] and in vivo bone formation.[19] Endothelial cell and osteoblast coculture models reveal cell contact dependent changes in gene expression pattern in both cell lines, including up regulation of VEGF receptor 2 in endothelial cells and up regulation of alkaline phosphatase in osteoblasts.[12,13] It is clear, however, that endothelial cells improve human bone marrow stromal cell differentiation through direct cell contact.[21] The mechanism of endothelial assisted increase in osteogenesis in engineered tissue is not fully understood. This provides an exciting area of research in bone tissue engineering with an eventual goal of minimizing the need for autologous tissue.

Our results show that ASC ASC Endo cells will not increase bone formation in PLA scaffold by itself but can be used as a potential cell source for improving vascularization. The presence of osteogenic cells in a critical size defect, whether they be undifferentiated stem cells or osteoblastic cells, is necessary for bone formation.

CONCLUSIONS

Adipose derived stem cells were successfully differentiated in vitro into endothelial cells and osteoblasts in gas plasma treated PLA scaffolds using endothelial differentiation media with VEGF and osteoblast differentiation media containing BMP 2, respectively. There is increased vascularization in a critical size rat calvarial defect when endothelial differentiated ASCs are seeded in a gas plasma treated PLA scaffold, although it did not reach statistical significance. This increase in vascularization does not translate into increased bone formation and may in fact be inhibitory. To hasten bone regeneration, a source of osteogenic cells or osteogenic precursor cells within the scaffold graft is necessary. Further augmentation of bone regeneration may be possible through the use of both endothelial and osteogenic cells to supply the sources for vessel formation and osteogenesis, respectively, in a coculture system.

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